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January 2010

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### Recommended Citation

Nathan, Britto P.; Gairhe, Salina; Nwosu, Ikemefuna; Clark, Stephen; and Struble, Robert G., "Reconstitution of the Olfactory Epithelium Following Injury in ApoE-Deficient Mice" (2010). *Faculty Research & Creative Activity*. 186.  
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Published in final edited form as:

*Exp Neurol.* 2010 November ; 226(1): 40–46. doi:10.1016/j.expneurol.2010.08.001.

## Reconstitution of the Olfactory Epithelium Following Injury in ApoE-Deficient Mice

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### Abstract

ApoE, a protein component of lipoproteins, is extensively expressed in the primary olfactory pathway. Because apoE has been shown to play a vital role in nerve repair and remodeling, we hypothesized that apoE expression will increase in the injured olfactory epithelium (OE), and that apoE deficiency in apoE knockout (KO) mice will lead to delayed/incomplete reconstitution of the OE following injury. To directly test this hypothesis, we compared OE regeneration in wild-type (WT) and KO mice following injury induced by intranasal irrigation of Triton X-100. OE was collected at 0, 3, 7, 21, 42, and 56 days post lesion. The amount and distribution of apoE in the regenerating OE was measured by immunoblotting and immunohistochemistry. Rate of OE reconstitution in WT and KO mice was assessed by using three independent measures: (1) OE thickness was measured in cresyl-violet stained sections, (2) basal cell proliferation was determined by using bromodeoxyuridine (BrdU) staining, and (3) differentiation and maturation of olfactory sensory neurons were measured by immunoblotting and immunohistochemical analysis of growth associated protein (GAP) 43 and olfactory marker protein (OMP). The results revealed that apoE expression in the OE is highly regulated during the entire course of OE reconstitution post injury, and that apoE deficiency in apoE KO mice leads to delayed recovery of mature OMP<sup>+</sup> cells in the reconstituting OE. The data suggest that apoE production increases in the injured OE to facilitate maturation of olfactory sensory neurons.

### Keywords

Apolipoprotein E; plasticity; Alzheimer's disease; regeneration; olfactory

### Introduction

Apolipoprotein E (apoE), a 35 kDa lipoprotein associated protein, has been shown to play a major role in neuronal regeneration and repair (Fullerton et al., 1998; Ignatius et al., 1986; Ignatius et al., 1987a; Ignatius et al., 1987b; LeBlanc and Poduslo, 1990; Mahley, 1988; Nathan

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et al., 2001; Snipes et al., 1986). In a variety of injury paradigms, apoE expression increases post injury. For instance, apoE protein increased by 250- to 350-fold three weeks post crush induced injury of peripheral nerve in rats (Ignatius et al., 1986; Ignatius et al., 1987a; Ignatius et al., 1987b). The increased apoE production at the injury site has been proposed to scavenge lipid from the degenerating neurons and provide it to growth cones of sprouting axons through the lipoprotein receptors (Mahley, 1988). Consistent with this postulate, *in vitro* studies have demonstrated that apoE containing lipoproteins stimulate neurite outgrowth from a variety of neurons in culture (Bellosta et al., 1995; Holtzman et al., 1995; Nathan et al., 1994; Nathan et al., 1995; Nathan et al., 2002; Nathan et al., 2004; Teter et al., 1999; Teter et al., 2002). Together, these data suggested that apoE is important for nerve regeneration, and has led to the hypothesis that absence of apoE in apoE-gene deficient/knockout (KO) mice would lead to reduced and perhaps aberrant regeneration following injury.

Several studies have compared nerve regeneration process following injury in apoE KO with wild-type (WT) mice. Axonal degeneration and myelin sheath alterations were observed in the optic nerve of KO mice (Lopez-Sanchez et al., 2003). Also, KO mice had abnormal and reduced numbers of unmyelinated axons within the sciatic nerve (Fullerton et al., 1998). Furthermore, ischemia induced brain damage was considerably higher in KO compared to WT (Horsburgh et al., 1999). ApoE may be important in CNS plasticity, although data are equivocal. A study found fewer synapses, vacuolated and swollen dendrites, and a reduced recovery following perforant pathway lesioning in KO mice as compared to controls (Masliah et al., 1995). In addition, KO animals displayed impaired spatial learning that could be corrected by infusion of purified human apoE3 (one of the three isoforms of human apoE) into the lateral ventricles (Masliah et al., 1997). In contrast to the above findings, peripheral nerve regeneration following sciatic nerve injury occurred equally as well in KO mice as compared to WT (Anderson et al., 1998; Gandy, 1995). Thus the importance of apoE in nerve regeneration and repair is controversial, and warrants further studies.

The adult olfactory system, with its exceptional ability to regenerate olfactory sensory neurons (OSN) from stem cells throughout the life of the organism, makes it an important neural system for the study of mechanisms functioning in neuronal degeneration and post-lesion plasticity. Results from our previous studies revealed that apoE levels in the olfactory bulb (OB) increased two-fold at 3 days post-OE injury induced by nasal irrigation of Triton X-100 (TX) in mice (Nathan et al., 2001). ApoE levels remained elevated by approximately 1.5 times normal levels at 7 through 21 days after injury and returned to baseline by 56 days.

Immunohistochemical studies revealed that both astroglia and microglia in the OB increased apoE production following OE injury. Comparison of nerve regeneration in WT and KO littermates following OE lesioning has suggested a vital role for apoE in OSN plasticity (Nathan et al., 2005). In KO mice the decline of bulb olfactory marker protein (OMP; marker for mature OSN) post-OE injury was prolonged and the onset of nerve recovery was delayed, as compared to WT littermates. How apoE deficiency leads to delay in the olfactory nerve recovery in KO mice is unknown.

The present study was designed to examine three potential mechanisms whereby apoE may support OE reconstitution following injury: (1) apoE facilitates injury-induced cell proliferation in the OE, (2) apoE supports differentiation of newly generated basal cells to neurons, and (3) apoE promotes axonal growth and maturation of OSN in the injured OE. We found that apoE expression in the OE is highly regulated during the entire course of OE reconstitution post injury, and that apoE deficiency in KO mice leads to delayed recovery of mature OMP<sup>+</sup> cells in the reconstituting OE. The data suggest that apoE increases in the injured OE to facilitate OSN maturation.

## Materials and Methods

### Animals

Breeding pairs of homozygous KO mice that had been backcrossed at least 10 generations to the C57BL/6 strain and C57BL/6 WT controls were purchased (Jackson Laboratory, Bar Harbor, ME). ApoE genotype of the litters were verified by PCR and confirmed by immunoblotting using anti-apoE (1:1000; Calbiochem, San Diego, CA) as described (Masliah et al., 1996).

### OE Lesion

Two- to four-month-old male KO and WT mice were lesioned as previously described (Margolis et al., 1974; Rochel and Margolis, 1980; Verhaagen et al., 1990b). Briefly, a 25-gauge needle with a rounded tip, was inserted about 2 mm into the nostril, and 50  $\mu$ l of 0.7% (v/v) Triton-X100 (TX) (Sigma, St. Louis, MO) in saline, or 50  $\mu$ l of saline alone (SL, control) was infused into a nostril of mice. This procedure was repeated on the other nostril. The excess solution was drained from the nasal passages by gently shaking the mice. This technique results in extensive bilateral damage to the OE with approximately 70–80% of the adult OE lesioned (Nathan et al., 2001). This procedure, however, spares the basal cells, which subsequently divide and differentiate into new OSN (Margolis et al., 1974; Rochel and Margolis, 1980; Verhaagen et al., 1990b).

### Tissue Preparation

Following the survival periods (below) after TX- or SL-irrigation, mice were anesthetized with pentobarbital (80 mg/kg), and prepared for either immunoblotting or immunohistochemistry. Mice were sacrificed at days 0, 3, 7, 21, 42, and 56 days post-treatment. Six mice were used per time point.

For immunoblotting analysis, mice were perfused transcardially with 0.9% saline until the perfusate was free of blood. OE were dissected, washed with ice cold 0.1M phosphate buffered saline (PBS, pH=7.4), and processed for immunoblotting as described below. Statistical analysis of immunoblotting data were performed on three mice for a given time point and condition within the same immunoblot.

For histology, mice received an intraperitoneal injection of bromodeoxyuridine (BrdU) (50 mg/kg) (Sigma Aldrich, St. Louis, MO) 12 hours prior to sacrifice. For fluorescence immunohistochemistry, mice were anesthetized as described above and transcardially perfused with cold saline (0.9% NaCl), followed by 4% paraformaldehyde in 0.1M PBS. Olfactory turbinates were removed and cryoprotected overnight in 30% sucrose in 0.1 M PBS. After cryoprotection, the turbinates were frozen with dry ice and sections were cut on a cryostat at 18  $\mu$ m, and air dried for 2 h at room temperature.

### Immunoblotting

Quantitative immunoblotting for OE levels of apoE, OMP, and GAP43 was performed as described (Bellosta et al., 1995; Nathan et al., 1995). Briefly, OE were homogenized in 100  $\mu$ l of ice-cold TMN buffer (25 mM Tris-HCl [pH 7.6] 3 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride). The homogenate was lysed by adding 20 % Triton X-100, 10 % deoxycholate, and 10 % SDS on ice for 5 min. The homogenate was then centrifuged (10,000  $\times$  g) for 2 min, and 20  $\mu$ g of supernatant was mixed with an equal volume of 2 $\times$  Laemmli sample buffer and heated at 100  $^{\circ}$ C for 5 min. The proteins in the supernatant were resolved by 10–20% gradient SDS-PAGE and transferred on to Immobilon PDVF membrane (Fisher, St. Louis, MO) using a wet trans-blot transfer cell (Bio-Rad, Hercules, CA) following the manufacturer's procedure. Blots were rinsed in T-TBS (0.1 M Tris, 0.15 M NaCl, 0.1% Tween-20), and

incubated with goat anti-apoE (1:1,000, Calbiochem, San Diego, CA), goat anti-rat OMP (1:7,000 dilution, Wako, Richmond, VA) or with monoclonal mouse IgG against rat GAP43 (1:7000, Sigma, St. Louis, MO) in T-TBS for 1 h on a shaker at room temperature. Following incubation with primary antibodies, the blots were washed six times (5 min each) in T-TBS, and incubated with donkey anti-goat (for apoE and OMP), or with goat anti-mouse (for GAP43) secondary antibody conjugated to HRP (Jackson Immuno Research Laboratories) at 1:1,000 dilution in T-TBS for 30 min at room temperature. Immunoreactivity was visualized with Pico Chemiluminescence kit (Pierce, Rockford, IL), and the optical density (OD) of the immunoreactive bands were quantified by Scion image analysis system (Scion Image, Frederick, MD). To confirm antibody specificity, immunoblots were performed with a 30-fold excess of normal serum of the species in which the primary antisera was obtained.

For actin immunoblotting, the blots were soaked for 1h at room temperature in stripping buffer (Pierce, Rockford, IL), washed in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 0.1 g BSA, pH 7.5), and incubated for 1h at room temperature with mouse anti-actin (Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA) at 1:10,000 dilution in TBST. Blots were washed with TBST, and incubated for 1h at room temperature in HRP conjugated goat anti-mouse IgG (Chemicon, Temecula, CA) at 1:10,000 dilution in TBST. Blots were washed in TBST and bands visualized as previously described for apoE. All experiments were repeated at least three times to assure reproducibility of the results.

### CV Staining

Sections were rinsed in distilled water for 10 minutes and placed in the oven for 2 hours at 37° C. The sections were then defatted with xylene for 30 minutes. The sections were hydrated in a descending concentration of ethanol (100%, 95%, and 70%) for 10 minutes each. Sections were rinsed in water and stained in cresyl violet acetate solution (Sigma, St. Louis, MO) for 4 minutes. Sections were rinsed in water and dehydrated in an ascending concentration of ethanol (70, 95, and 100%). Following incubation in xylene for 30 minutes, sections were cover-slipped using permount (Fisher Scientific, Fair Lawn, NJ).

### Immunohistochemical Analysis

Sections on slides were rinsed in 0.1 M PBS, and permeabilized with 0.2 % Triton X-100 (Sigma, St. Louis, MO) in PBS for 30 minutes at room temperature. The slides were rinsed once with PBS and treated with 70, 90, 100, 90, and 70% ethanol for two minutes each (Jang et al., 2003). Non-specific immunoreactivity was attenuated by incubation in 2.25% gelatin in 0.1 M PBS for 1 h, followed by overnight incubation with primary antisera solution at 4°C (see Table 1 for source and concentration used). The sections were washed three times in PBS, and incubated for 1 hour at room temperature with secondary antibody solution as listed in Table 1. The sections were washed three times in PBS, mounted in Vectashield (Vector labs, Burlingame, CA). Specificity was determined by incubation with normal serum in place of the primary antisera which resulted in no staining.

Stained sections were examined using an Olympus BX-50 microscope. Images were captured using a Pixera Digital Camera (Pixera, Los Gatos, CA) and saved as high resolution TIF files. Figures from images were assembled using Photoshop (Adobe, San Jose, CA). Image analysis was performed using Scion Image software (Scion Image, Frederick, MD).

Morphological thickness of the OE was determined from image calibration of a stage micrometer in Scion Image. Thickness was repeatedly measured from the horizontal basal cell layer to the head of the sustentacular cells. The number of OMP<sup>+</sup>, GAP43<sup>+</sup>, and BrdU<sup>+</sup> cells in ten 100 µm segments of OE was measured using Scion Image.

## Statistical Analysis

A total of 10 measurements were taken from each animal. The data in individual experiments were presented as mean  $\pm$  standard error and statistical analysis (ANOVA, Repeated Measures ANOVA) was performed using SYSTAT. A blinded procedure was employed in all experiments so the experimenter was unaware of the genotype (WT versus KO) and treatment (saline versus triton) received by the animals.

## Results

### ApoE

Results from the immunoblotting studies revealed that expression of apoE in the OE declined sharply following nerve lesioning, decreasing to about 1/3 normal levels at 7 days post lesion (Fig. 1). Thereafter, the apoE level increased gradually to about normal level by 21 days post lesion. ApoE level continued to increase reaching its peak at 42 days post lesion, and at 56 days it declined, but still stayed well above initial levels. Statistical analysis of apoE levels in the OE was performed with a one-way ANOVA on days post lesion. Days post lesion was significant ( $F_{6,14}=144.55$ ;  $p<0.001$ ). Post-hoc testing disclosed that apoE declined from initial levels after TX treatment at seven days returned to “normal” levels at 21 days and then significantly exceeded initial levels at days 42 and 56.

Immunohistochemical studies of saline irrigated mice displayed the previously described staining pattern of apoE in the OE and its underlying lamina propria (Nathan et al., 2007; Yamagishi et al., 1998). ApoE immunostaining was observed mainly in the sustentacular cells, with strong staining in its perikarya, located in the epithelial surface, and its endfeet, surrounding the basal cells (Fig. 2). In the lamina propria, apoE immunoreactivity was intense in the endothelial cells of the blood vessels, and diffuse in the olfactory fascicles. Following lesion, the shrinkage of the OE was clearly evident at 1 and 3 days post-lesioning. During this degeneration phase, apoE expression increased in the basal cell layer of the OE, and in the endothelial cells and olfactory fascicles in the lamina propria. Starting 7 days post lesion, the OE starts to recover. During this regeneration phase, apoE immunoreactivity is diffuse throughout the OE, and it gradually increased in intensity, attaining its peak expression at 42 days post lesion. At 56 days, apoE staining pattern is similar to SL irrigated mice, but more intense than the SL irrigated mice. Together the results from the immunoblotting and immunohistological studies revealed that apoE expression modulates during the entire course of the olfactory nerve regeneration, and suggest a role for apoE in reconstitution of the OE post-lesioning.

### CV

We next examined the time course of recovery of OE thickness in WT and KO mice by using CV staining (Fig. 3). At 3 days post-lesioning, the thickness of the OE in both genotypes was reduced to 1–2 layers of cells above the basal lamina. Thereafter, the OE thickness increased gradually to near normal thickness by 56 days post lesion. Statistical analysis of the OE thickness data was performed with a two-way analysis of variance with factors of days after lesion, and genotype (WT or KO mice). Analysis found a significant effect of day following lesion ( $F_{7,32}=311.70$ ;  $p<0.001$ ) and of genotype ( $F_{1,32}=13.22$ ;  $p<0.001$ ). Importantly, no interaction between days following lesion and genotype was found ( $F_{7,32}=1.133$ ; N.S.) emphasizing that the magnitude of the lesion effect and rate of recovery were comparable in the two genotypes. Overall, thickness of the OE in the KO mice was thinner than that of the WT mice. As expected, OE thickness declined following TX-treatment and then recovered. Thus, this analysis suggests that OE is thinner in KO versus WT mice; however, recovery of the OE thickness was equivalent in both genotypes.



## BrdU

We next examined if apoE deficiency would impede proliferation of basal cells in the OE by comparing BrdU<sup>+</sup> cell count in WT and KO mice. The data revealed that there was a clear effect after the lesion ( $p<0.000$ ); essentially, BrdU<sup>+</sup> cell count sharply increased at day 3 in both WT and KO, and then declined thereafter (Fig. 4). There was, however, no difference in BrdU<sup>+</sup> cells by genotype or in the interaction between day and genotype. In essence, deficiency of apoE in KO mice did not affect basal cell proliferation.

## GAP43

Given the established role of apoE in promoting neurite outgrowth, we examined the effect of apoE deficiency on GAP43, a protein associated with olfactory nerve recovery following lesion (Verhaagen et al., 1989; Verhaagen et al., 1990a). Analysis of TX-treated groups showed a genotype ( $F_{1,20}=6.46$ ;  $p<0.016$ ) and days post lesion effect ( $F_{4,20}=163.07$ ;  $p<0.001$ ), but no interaction ( $F_{4,20}=1.86$ ; NS) (Fig. 5). Statistical analysis revealed that, GAP43<sup>+</sup> cells in WT mice was at an overall higher level than that in KO mice regardless of days after lesion. Following lesion, GAP43<sup>+</sup> cell counts reached their highest level at 21 days post lesion in both genotypes. Post hoc testing showed a significantly higher level even at 56 days post lesion.

## OMP

OMP level in the OE following injury is a reliable marker of OSN maturation. We employed both immunoblotting and immunohistochemistry to examine the effects of apoE deficiency on maturation of OSN following injury. Immunoblotting showed an interaction between day post lesion and genotype ( $F_{6,28}=4.64$ ;  $p<0.002$ ) (Fig. 6). Post hoc pair-wise analysis showed that baseline levels and those three days following TX treatment were not different between the two groups. In contrast, the concentration of OMP in OE was less in the KO than in the WT at days 21 and 42 with a likely difference at day 14. Western blotting clearly demonstrated that OSN maturation is delayed in KO mice.

Immunohistochemical data for OMP confirmed the immunoblot data. In SL-treated mice, OMP immunoreactivity was localized in 6–7 layers, with no clear differences between WT and KO mice (Fig. 7). ANOVA identified a significant interaction between genotype and day after lesion ( $F_{7,32}=2.93$ ;  $p<0.017$ ). Post-hoc testing showed comparable cell counts at 0, 3 and 7 days post TX-treatment. Most of the OMP<sup>+</sup> cells have degenerated at three and seven days. Recovery of OMP<sup>+</sup> cells is evident from 14–56 days post injury in both genotypes; however, OMP<sup>+</sup> cells were fewer in KO mice than WT mice. In essence these data showed that the conversion of immature OSN to an adult phenotype (i.e., OMP<sup>+</sup>) was delayed in the KO mice.

## Discussion

The present study demonstrates that apoE expression in the OE is highly regulated during the entire course of OE reconstitution post injury. In addition, the results revealed that apoE deficiency in KO mice leads to delayed recovery of mature OMP<sup>+</sup> cells in the reconstituting OE, suggesting that the apoE increase in the injured OE facilitates OSN maturation.

ApoE staining in the OE of SL-irrigated mice in this study replicate previous reports and shows apoE expression throughout the OE and its underlying lamina propria (Nathan et al., 2007; Yamagishi et al., 1998). In the OE, sustentacular cells express high levels of apoE in their perikarya and villar processes, located in the surface of OE, and in their endfeet, which are in close proximity to basal cells located above the basal lamina. In the lamina propria, apoE is expressed at high levels by the endothelial cells of the blood vessels, and putative ensheathing cells of the olfactory fascicles. Results from the present study clearly demonstrate that apoE expression is highly modulated in the OE following injury. Three days post lesion, total apoE

levels in the OE increased slightly, as measured by immunoblotting. Immunohistochemical studies revealed that apoE immunoreactivity on 1 and 3 days post injury was intense in the basal cell layer, in the endothelial cells of the blood vessels, and in the olfactory nerve fascicles. The increased apoE production in the OE during these early stages of repair could probably scavenge lipid from degenerating cells, and thus aid in removal of debris. Previous studies on KO mice have shown accumulation of degeneration products following entorhinal cortical lesion (Fagan et al., 1998). Another potential function of the apoE at this early stage is to provide lipid for membrane biosynthesis to support basal cell proliferation. The apoE level increased gradually to about normal level by 21 days, and reached peak levels at 42 days post lesion. One possible function of apoE during this regeneration phase is to provide lipid to facilitate axonal growth of the OSN. Although apoE levels declined at 56 days post lesion, it still remained well above initial levels. It is possible that apoE increase observed in this study could be due to aging of mice, as the study progressed for two months. This latter possibility has to be examined in future studies.

Although apoE is clearly regulated during degeneration and regeneration, apoE does not appear to be experimentally critical for OE reconstitution post injury. Our analysis of basal cell division (BrdU), differentiation of newly generated basal cells to immature OSN (GAP43), and ultimately the thickness (CV) of the OE did not identify any evidence of modification of these processes by the absence of apoE. The logical conclusion of this pattern of observations is that apoE, although regulated, is not critical in local repair of the OE.

However, OMP, a marker of adult OSN, is markedly delayed by the absence of apoE. This observation suggests that apoE may be more critical in promoting maturation of the newly differentiated OSN in the injured OE. This postulate is consistent with previous studies where apoE has been shown to increase neurite outgrowth in a variety of cell culture paradigms (Bellosta et al., 1995; Holtzman et al., 1995; Nathan et al., 1994; Nathan et al., 1995; Nathan et al., 2002; Nathan et al., 2004; Teter et al., 1999; Teter et al., 2002). Given that apoE is the only apoprotein in the CNS that can interact with lipoprotein receptors, apoE has a critical role in lipid recycling in the injured CNS (Mahley, 1988). However, in the peripheral nervous system, other apoproteins such as apoB and apoA may be able to functionally substitute apoE to clear of degeneration products resulting from an injury. However, in the CNS, OSN cells are delayed in making synapses, which impedes maturation of OSN to adult phenotype. Another possibility is that apoE supports the survival of the newly generated OSN by an unknown mechanism. This latter possibility has to be examined in future studies.

It is interesting to note that olfactory function shows early severe deficits in numerous chronic neurodegenerative disorders where apoE4 genotype is a major risk factor (Ansari and Johnson, 1975; Hawkes, 2006; Murphy et al., 2009; Olofsson et al., 2009; Talamo et al., 1989). In light of the results from this study, and other previous studies that has documented a critical role for apoE in nerve repair, we propose that olfactory dysfunction observed in various neurological diseases is a manifestation of inefficient lipid recycling due to the expression of the apoE4 isoform.

## Acknowledgments

This work was supported by National Institute on Deafness and Other Communication Disorder (DC 003889), Illinois Department of Public Health grant, and Eastern Illinois University CFR grants.

## Abbreviations

|      |                  |
|------|------------------|
| ApoE | apolipoprotein E |
| WT   | wild-type        |



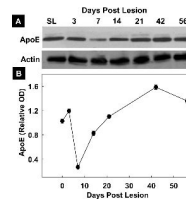
|       |                              |
|-------|------------------------------|
| KO    | knock-out                    |
| OB    | olfactory bulb               |
| ON    | olfactory nerve              |
| OE    | olfactory epithelium         |
| OSN   | olfactory sensory neurons    |
| GAP43 | growth associated protein 43 |
| OMP   | olfactory marker protein     |
| AD    | Alzheimer's disease          |
| CNS   | central nervous system       |
| PNS   | peripheral nervous system    |

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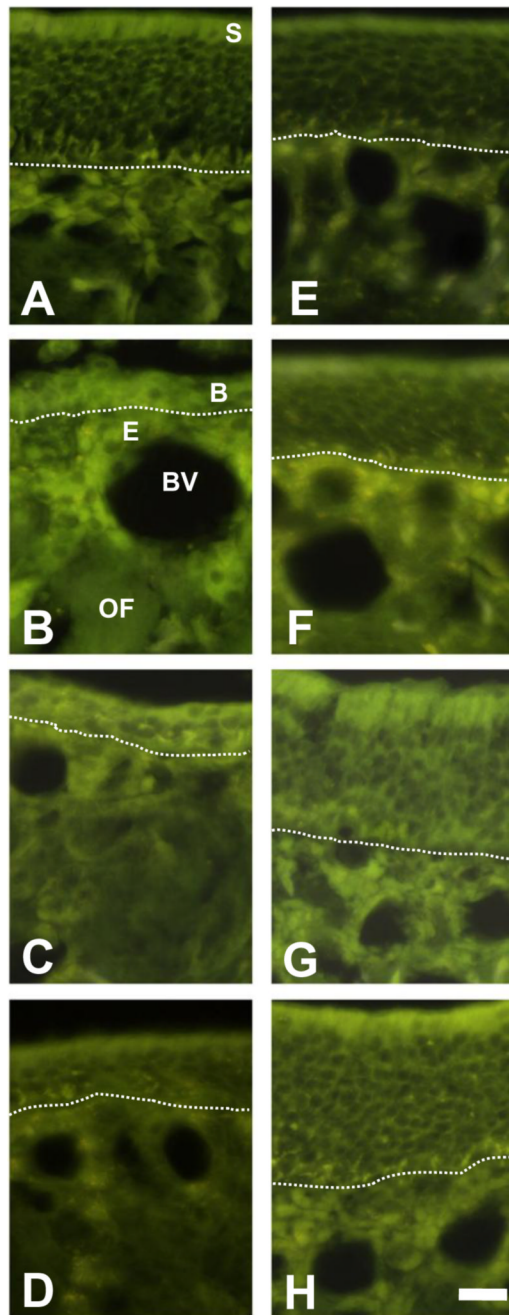
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**Fig. 1.**

Immunoblotting of apoE in the OE following lesioning in mice. (A) OE homogenates collected at indicated times from WT mice following nasal irrigation of TX or saline (SL) were immunoblotted using a polyclonal antibody to apoE as described under Materials and Methods. Actin was used as internal standard for protein loading. (B) The relative changes (optical density; means  $\pm$  SE) in apoE levels post- nasal irrigation. Days post lesion was significant ( $F_{6,14}=144.55$ ;  $p<0.001$ ). All data points were generated by densitometric scans of immunoblots in three separate experiments.

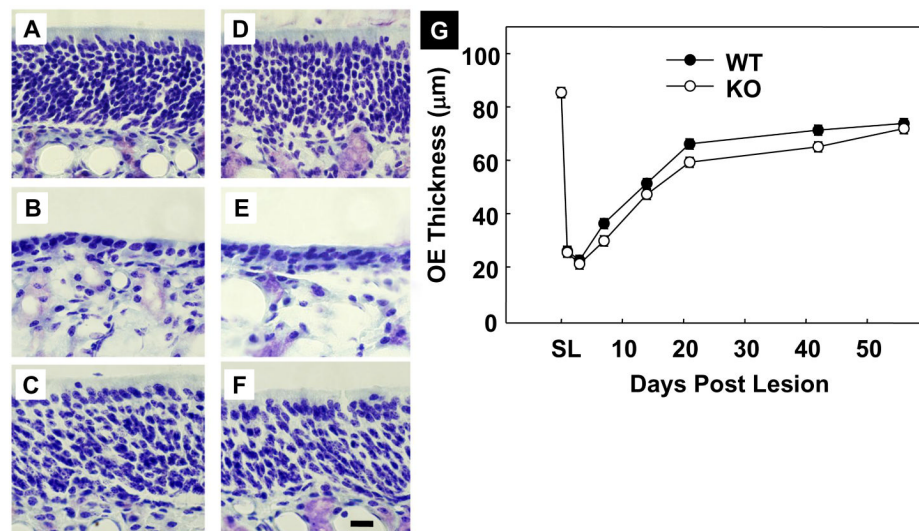


**Fig. 2.**

ApoE immunoreactivity in the OE following nasal irrigation. In the SL irrigated mice (A), apoE immunoreactivity was observed in the perikarya of sustentacular cells (S). At 1 (B) and 3 (C) days post TX-lesioning, the thickness of OE decreased drastically as compared to SL irrigated mice (A). ApoE immunoreactivity during this degeneration phase was intense in the basal cell layer (B), the endothelial cells (E) of the blood vessels (BV), and in the olfactory nerve fascicles (OF). Recovery of OE thickness is apparent at 7 days (D), gradually proceeds on days 14 (E), 21 (F), and 42 (G), and reaches normal level by 56 days (H). ApoE level during this regeneration phase simultaneously increases with increase in OE thickness, reaching its

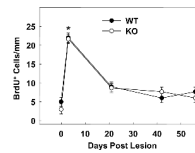
peak at 42 days, followed by a gradual decrease to basal level by 56 days. Basal lamina is indicated by the dotted line. Scale bar = 25  $\mu\text{m}$ .





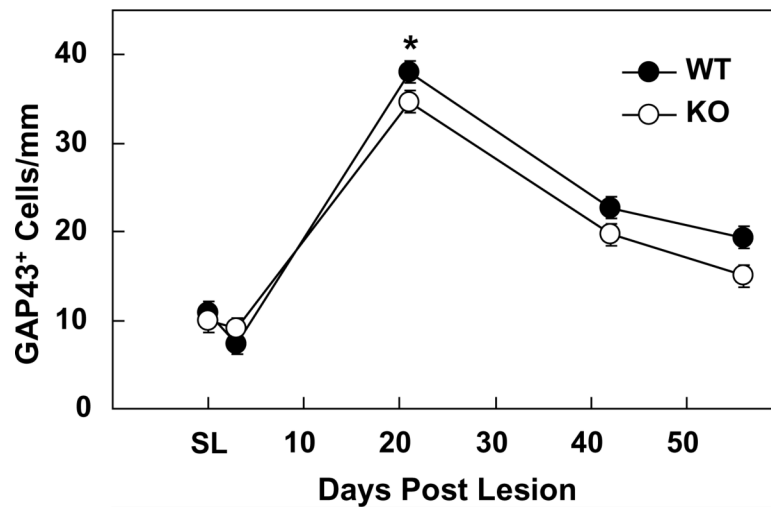
**Fig. 3.**

CV staining of OE following nasal irrigation. WT mice are shown on the left (A–C), and KO mice are shown on the right (D–F). No clear differences between WT and KO mice are apparent in SL-treated mice (A, D), or at 3 (B, E) and 56 (C, F) post-lesioning. The scale bar = 25  $\mu\text{m}$ . G. Relative changes in OE thickness are shown in this graph as a function of days post-lesion. At days 3 and 7 post-lesion, the OE thickness drastically decreased in both WT and KO mice. Starting from 7 days post lesion, the OE thickness gradually recovers to roughly normal levels by 56 days in both WT and KO mice. Overall, the OE thickness was thinner in the KO mice than that of the WT mice at all time points; however, no significant difference in rate of recovery of OE was evident between WT and KO mice.



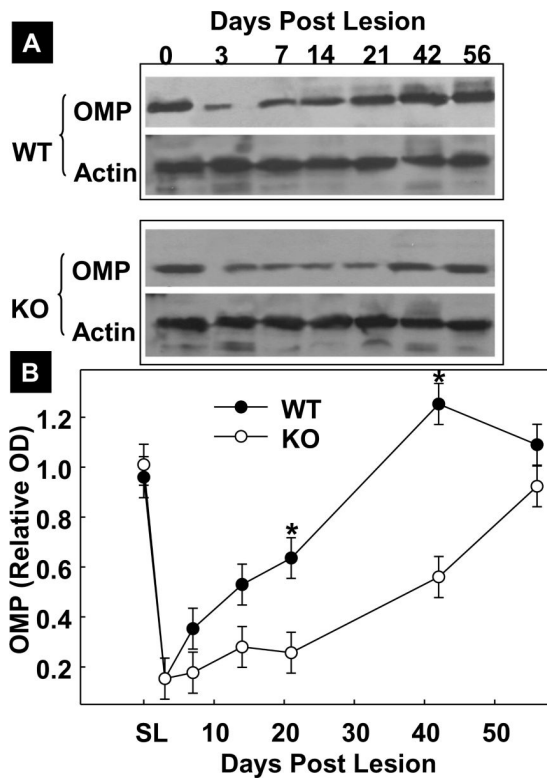
**Fig 4.**

BrdU<sup>+</sup> cells in the basal cell layer of the OE in WT and KO mice are shown as a function of days following lesion. OE lesioning induced a rapid surge in BrdU<sup>+</sup> cells in the basal cell layer in both WT and KO mice, and it peaked at 3 days post-lesion (\* 0 days versus 3 days post lesion,  $p < 0.000$ ). Following this initial surge, BrdU<sup>+</sup> cell numbers quickly returned to roughly normal levels by 21 days post lesion. No significant differences between WT and KO mice were observed.

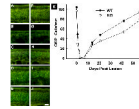


**Fig 5.**

GAP43 immunoreactive cell density in the OE of WT and KO mice following lesion. In both WT and KO mice, GAP43<sup>+</sup> cell counts increased after lesion, reaching their highest level at 21 days post lesion ( $F_{4,20}=163.07$ ;  $p<0.001$ ). Thereafter, the GAP43<sup>+</sup> cell counts gradually declined, but never reached basal levels even on day 56 post lesion. Overall, KO mice had lower levels of GAP 43<sup>+</sup> cells than WT mice ( $F_{1,20}=6.46$ ;  $p<0.016$ ).

**Fig. 6.**

Time course of OMP in OE following lesioning in mice. (A) OE homogenates collected at indicated times from WT and KO mice following nasal irrigation of TX or saline (SL) were immunoblotted using a polyclonal antibody to OMP as described under Materials and Methods. Actin was used as internal standard for protein loading. (B) The relative changes (optical density; means  $\pm$  SE) in the OMP levels were generated by densitometric scans of immunoblots in three separate experiments. OMP level in OE was significantly less in the KO than in the WT at days 21 and 42 days post lesion (\*  $p < 0.001$ ).



**Fig. 7.**

OMP immunoreactivity in the OE following TX lesioning in WT and KO mice. WT mice are shown in the left, and KO mice are shown on the right. No clear differences in the OMP<sup>+</sup> cell counts are apparent in SL irrigated (A, F) mice. At 3 (B, G) days post TX-lesioning, the number of OMP<sup>+</sup> cells in both WT and KO mice decreased drastically as compared to SL irrigated mice (A, F). Recovery of OMP<sup>+</sup> cells is evident from 14 days post lesion in both WT and KO. However, OMP<sup>+</sup> cell density of KO mice was lower than WT on days 14 (C, H), 42 (D, I) and 56 (E, J) days post-lesion. Scale bar = 25  $\mu$ m. K. Relative changes in OMP<sup>+</sup> cell counts in the OE of WT and KO mice are shown in this graph as a function of days post TX lesion. ANOVA identified a significant interaction between genotype and day after lesion ( $F_{7,32}=2.93$ ;  $p<0.017$ ).

**Table 1**

List of primary and secondary antibodies used in this study

| Antibodies        | Host   | Source                    | Dilution |
|-------------------|--------|---------------------------|----------|
| ApoE              | Goat   | Calbiochem, San Diego, CA | 1:1000   |
| OMP               | Goat   | Wako, Richmond, VA        | 1:500    |
| GAP43             | Rabbit | Chemicon, Temecula, CA    | 1:500    |
| BrdU              | Rat    | Accurate, Westbury, NY    | 1:500    |
| FITC-anti goat    | Donkey | Jackson, West Grove, PA   | 1:500    |
| Cy3-anti goat     | Donkey | Jackson, West Grove, PA   | 1:500    |
| Cy3-anti rat      | Goat   | Jackson, West Grove, PA   | 1:500    |
| Alexa-anti rabbit | Donkey | Invitrogen, Eugene, OR    | 1:200    |